INHIBITOR SPECIFICITY OF AMINE OXIDASE

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In their studies of the oxidation of adrenalin and other amines by amine oxidase preparations, Blaschko, Richter, and Schlossmann (1) found that *l*-ephedrine, triethylamine, triisoamylamine, and *dl*-1-hydroxy-2-hydrindeneamine are not only not oxidized, but inhibit the action of the enzyme upon isoamylamine. The conclusion was drawn that this inhibition was of the competitive type, because some data were available which indicated a dependence of degree of inhibition upon the concentration of both enzyme and substrate. Natural *l*-ephedrine and the related compounds, suprifen and veritol, were later reported by Blaschko (2) to inhibit amine oxidase in its action upon *l*-sympathol. Some further extension of such studies to include benzedrine, and the finding that this compound and veritol are stronger inhibitors of amine oxidase than are ephedrine isomers in the particular system studied, led Blaschko (3) to conclude there was agreement between the inhibiting effects *in vitro* on amine oxidase and their awakening properties in animals which are pharmacological effects upon the central nervous system.

Previous to this, Gaddum and Kwiatkowski (4) had advanced the theory that ephedrine action in animals was due to its inhibition of adrenalin oxidation by amine oxidase in the body. This was based on demonstration that in the perfused rabbit ear, sympathetic stimulation liberated a substance indistinguishable from adrenalin, and that this substance like adrenalin is increased in certain of its physiological activities by addition of ephedrine. The study by Richter and Tingey (5) of the kinetics of the rate of inactivation of adrenalin by amine oxidase and the degree of inhibition of this oxidation by ephedrine permitted calculations which did not support the view that amine oxidase is specifically concerned in the inactivation of adrenalin in the body. Further evidence obtained by Richter (6) and Richter and MacIntosh (7) from their isolation of some of the metabolic products excreted in the urine after administration of adrenalin and other *o*-diphenolic pressor amines makes it quite certain that adrenalin is not considerably acted on by amine oxidase in passing through the body.

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Thus, although at the present time it appears clear that amine oxidase oxidation of adrenalin, or other *o*-diphenolic pressor amines such as were studied by Richter (6), does not play a significant physiological rôle, it is equally clear that the inactivation of aliphatic amines, phenethylamine and probably 4-hydroxyphenethylamine (tyramine), does predominantly take place by amine oxidase oxidation. In view of the evidence from the experiments of Ewins and Laidlaw (8) and a later study by Guggenheim and Löffler (9), such amine oxidations chiefly occur in the liver. In the present studies, an attempt was made to value quantitatively the inhibition of some of these particular type compounds by certain types of amines which are not themselves oxidized by the enzyme system (see Alles and Heegaard (10)).

EXPERIMENTAL

Purified amine oxidase preparations from rabbit liver were used throughout the present work. They were prepared as described by Alles and Heegaard (10) and experiments in which there was any considerable uptake of oxygen by the control alone were discarded along with that particular enzyme preparation. The hydrogen ion activity was maintained uniformly at pH 7.0 in all experiments, though it is expected that there can be observed a relation between pH and degree of inhibition. Such relation will probably have a considerable variation even among closely related series of compounds, just as the variation in amine oxidase activity with pH depends on the particular substrate acted upon, as shown by our studies (10).

It was most desirable to obtain data indicating inhibitor effects with a considerable number of compounds of different types, and then to make a critical study with a particular inhibitor and various substrate types to determine whether such inhibition was competitive.

The term observed activity in any experiment refers to the maximum rate of oxygen uptake. This often occurs only during the first few observations of any experiment, particularly with aliphatic amine substrates, and is determined as the tangent to the initial part of the plotted curve of observed oxygen uptakes. Such valuation of rate minimizes the effects of uncontrolled inhibiting variables that enter into rate valuations dependent upon a single observation made after some considerable time.

Aliphatic Amines As Inhibitors of Aliphatic Amine Substrates—Experiments were made to value the relative inhibiting effects of a series of secondary carbinamines. The oxidation system consisted of 2.0 ml. of purified amine oxidase preparation in 0.1 M phosphate buffer of pH 7.0, to which were added 0.1 ml. of 0.1 M amylamine as a neutral solution of its sulfate and 0.4 ml. of variable concentrations of inhibitor amine sulfate or chloride in neutral solution. The final concentration of amylamine in the mixture

was 0.004 M, and the concentrations of inhibiting amines were 0.004, 0.008, 0.012, and 0.016 M, giving the inhibitor-amylamine ratios presented in Table I. The oxygen uptake at 30° was followed with the Warburg apparatus and calculated to per cent of the uptake of the control sample with substrate but without any inhibitor.

The inhibiting effect in this series of data was clearly at a maximum with α-methylamylamine. In further experiments, in which the ratio of α-methylethylamine was 4:1, no inhibition was observed. α-Methyloctylamine and α-methylnonylamine were found to be less active inhibitors than α-methyl-

TABLE I

Inhibitor-Amylamine Ratios

In per cent of O_2 uptake, based on the rate without inhibitor.

Inhibitor / Amylamine	0	1:1	2:1	3:1	4:1
α-Methylpropylamine (*sec*-butylamine)	100	101	98	92	88
α-Methylbutylamine (*sec*-amylamine)	100	86	74	64	55
α-Methylamylamine	100	73	62	38	36
α-Methylhexylamine	100	90	73	53	42
α-Methylheptylamine	100	93	79	60	49

TABLE II

Effect of α-Methylbutylamine As Inhibitor

In per cent of O_2 uptake, based on the rate without inhibitor.

α-Methylbutylamine / Substrate	0	1:1	2:1	3:1	4:1
Butylamine	100	56	38	34	25
Amylamine	100	86	74	64	55
Hexylamine	100	91	84	83	79
Heptylamine	100	96	93	92	70

heptylamine. To cover a variance in substrate, α-methylbutylamine was studied as an inhibitor of several primary carbinamine substrates of 0.004 M final concentration in the oxidation system. The results are shown in Table II.

A series of tertiary carbinamines was studied under similar conditions as inhibitors of the primary carbinamine, butylamine. There appeared to be an increased inhibitor effect with larger molecular size. The results obtained with 4:1 inhibitor-substrate were as follows: α, α-dimethylethylamine (*tert*-butylamine) 100, α, α-dimethylpropylamine (*tert*-amylamine) 91,

α, α-dimethylbutylamine 84, α, α-dimethylamylamine 61, α, α-dimethylhexylamine 45.

Although Blaschko (11) noted, under his conditions of study, that cadaverine did not act as an inhibitor to the enzymic oxidation of amylamine or synephrine, observations were extended to include other aliphatic diamines. With 0.004 M amylamine as substrate and a 4:1 ratio of the ω-alkylenediamines, there was no exhibition of any notable inhibitor effect with tetramethylene-, pentamethylene-, hexamethylene-, heptamethylene-, and octamethylenediamine.

Another type of compound was of interest because of its relatively complete ionization in water solution. The alkyltrimethylammoniums were found not to be oxidized by amine oxidase in our earlier work (10). With 0.004 M amylamine as substrate and a 4:1 ratio of butyl-, amyl-, hexyl-,

TABLE III

Effect of α-Methylphenalkylamines As Inhibitor

In per cent of O_2 uptake, based on the rate without inhibitor.

Inhibitor / Amylamine	0	2:1	4:1
α-Methylphenmethylamine (α-phenylethylamine)	100	72	26
α-Methylphenethylamine (phenisopropylamine)	100	80	44
α-Methylphenpropylamine	100	70	20
α-Methylphenbutylamine	100	87	27
α-Methylphenamylamine	100	90	35

or heptyltrimethylammonium, no considerable inhibitor effect was observed.

Phenylaliphatic Amines As Inhibitors of Amine Substrates—While Blaschko (3) found that benzedrine (phenisopropylamine) and certain of its derivatives acted as inhibitors of amine oxidase under particular experimental conditions, his findings offered only meager experimental evidence for the generalization that inhibitor effect is a property of secondary carbinamines. A series of α-methylphenalkylamines was studied by us for their inhibitory effect on the oxidation of amylamine. The concentration of amylamine was 0.004 M, and other conditions were the same as in the studies of aliphatic amines as inhibitors (see Table III).

The inhibiting effect of certain types of derivatives of α-methylphenethylamine is of considerable interest because of possible relation to their physiological actions. A number of such derivatives (Table IV) were tested with amylamine as the substrate at 0.004 M and an inhibitor-amylamine ratio of 4:1.

Owing to a lack of time to complete more extensive studies, the observations of inhibition by secondary phenylaliphatic amines were extended to different amine substrates only with α-methylphenethylamine itself. Our previous determinations (10) of the kinetic dissociation constants of the enzyme-substrate complex of rabbit liver amine oxidase with aliphatic and phenylaliphatic amines indicated that these two types of compounds did not differ notably in their enzyme-substrate constants. Consequently, it was expected that any inhibitor would exhibit about the same degree of inhibition on these two types of substrates. Dissociation constants K_s for enzyme-tyramine and enzyme-hydroxytyramine had been found to be

TABLE IV

Effect of Methylphenethylamines As Inhibitor

In per cent of O_2 uptake, based on the rate without inhibitor.

Inhibitor / Amylamine	4:1	Inhibitor / Amylamine	4:1
α-Methylphenethylmethylamine	48	α-Methyl-3-hydroxyphenethylamine	65
α-Methylphenethylethylamine	39	α-Methyl-4-hydroxyphenethylamine	75
α-Methylphenethyldimethylamine	41	α-Methyl-4-hydroxyphenethylmethylamine	95
α,β-Dimethylphenethylamine	71	α-Methyl-4-methylphenethylamine	60
α-Methyl-β-hydroxyphenethylamine (*DL*-norephedrine)	97	α-Methyl-4-methoxyphenethylamine	64
α-Methyl-β-hydroxyphenethylamine (*DL*-norpseudoephedrine)	99	α-Methyl-3,4-dihydroxyphenethylamine	92
α-Methyl-β-hydroxyphenethylmethylamine (*DL*-ephedrine)	79	α-Methyl-3,4-dimethoxyphenethylamine	67
α-Methyl-β-hydroxyphenethylmethylamine (*DL*-pseudoephedrine)	85	α-Methyl-3,4-methylenedioxyphenethylamine	60
α,α-Dimethylphenethylamine	42	α-Methyl-3,4,5-trimethoxyphenethylamine	80
α,α-Dimethyl-β-hydroxyphenethylamine	79		

notably greater, and it was expected that these two amines would be more readily inhibited than either an aliphatic or phenylaliphatic amine. Simultaneous observations of the four types of amines in 0.004 M substrate concentrations alone and in the presence of α-methylphenethylamine (phenisopropylamine) gave the results shown in Table V.

The particular differences found in the effect with phenisopropylamine added to these different substrates were surprising, and some further studies were made to determine whether indeed competitive inhibition could be observed with phenisopropylamine and these different substrates.

Phenisopropylamine Inhibition of Amine Oxidase and Amines—Enzyme

properties are determined chiefly by means of kinetic studies. Competitive inhibition may be distinguished from other types primarily by conforming in its kinetics to the rate equations derivable from the equilibrium $2E + S + I \rightleftarrows ES$ (active) $+ EI$ (inactive) where E represents the enzyme, S the substrate, and I the inhibitor. In this simplest case, the rate relations can be represented in the reciprocal form by

$$\frac{1}{v} = \frac{1}{V_{\max.}}\left(K_s + \frac{K_s}{K_i}I\right)\left(\frac{1}{S}\right) + \frac{1}{V_{\max.}}$$

as given by Lineweaver and Burk (12). Here v is the reaction rate, $V_{\max.}$ the rate at infinitely high substrate concentration, S and I the molal concentrations of substrate and inhibitor, while K_s and K_i are the kinetic dissociation constants of the enzyme-substrate and enzyme-inhibitor compounds.

TABLE V

Effect of Phenisopropylamine As Inhibitor

In per cent of O_2 uptake, based on the rate without inhibitor.

Phenisopropylamine / Substrate	0	4:1	16:1
Amylamine	100	45	19
Phenethylamine	100	113	144
4-Hydroxyphenethylamine (tyramine)	100	26	11
3,4-Dihydroxyphenethylamine (hydroxytyramine)	100	20	13

Data were obtained to value the constants by choosing substrate concentrations as in the kinetic studies of our previous work with these same substrates (10). The enzyme concentration in the experiments was again about 0.5 enzyme unit per 2.5 ml. of total volume. Considerable difficulty was encountered in finding the suitable, but necessarily different, concentrations of phenisopropylamine for working with each substrate to get data of sufficient precision. These data were plotted as $1/v$ against $1/S$ so that the $1/v$ axis intercept is $1/V_{\max.}$ and the $1/S$ value of $2/V_{\max.}$ is $1/K_s$ or $1/(K_s + (K_s/K_i)I)$ in the presence of the inhibitor. From these values, which can be read directly from the graphs of Fig. 1, are calculated the values of $V_{\max.}$, K_s, and K_i.

It is immediately apparent from Fig. 1 that phenisopropylamine is many times more active an inhibitor with phenethylamine as the substrate than with amylamine. Similar experiments were carried at with tyramine and hydroxytyramine as the substrates, and the valuations of the enzyme constants are given in Table VI.

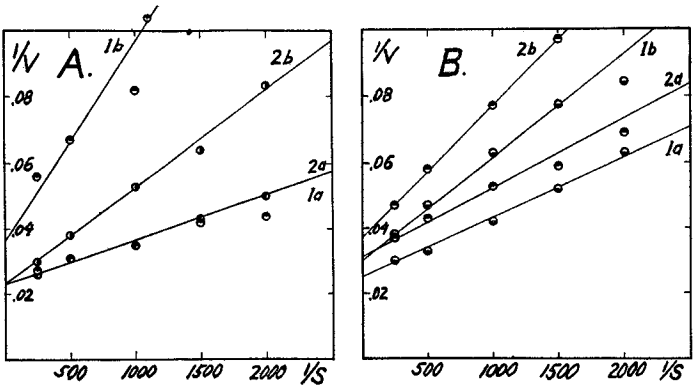

FIG. 1. Reciprocal microliters per 15 minute oxygen uptake rates against reciprocal molal substrate concentrations. At 30° with 2.0 ml. of purified rabbit liver amine oxidase in 0.2 M phosphate buffer, pH 7.0, made up with amine salt solution alone or with inhibitor amine salt to a total volume of 2.5 ml. *A*, Curves 1a and 2a, amylamine without added inhibitor; Curve 1b, amylamine and 0.0020 M phenisopropylamine; Curve 2b, amylamine and 0.0010 M phenisopropylamine; *B*, Curves 1a and 2a, phenethylamine without added inhibitor; Curves 1b and 2b, phenethylamine and 0.020 M phenisopropylamine.

TABLE VI

Enzyme-Substrate and Enzyme-Inhibitor Constants

Substrate used in variable concentration	Phenisopropylamine concentration	$\frac{1}{V_{max.}}$	$\frac{1}{K_s}$	$\frac{1}{K_s + \frac{K_s}{K_i} I}$	$V_{max.}$	K_s	K_i
	M				*microliters O_2 per min.*	*M*	*M*
Amylamine	0.0000	0.023	1650		42	0.00061	
"	0.0020	0.036		600	28		0.0011
"	0.0000	0.023	1650		42	0.00061	
"	0.0010	0.023		780	42		0.0009
Phenethylamine	0.0000	0.025	1360		40	0.00074	
"	0.0200	0.030		950	33		0.047
"	0.0000	0.031	1450		32	0.00069	
"	0.0200	0.037		920	27		0.035
4-Hydroxyphenethylamine	0.0000	0.031	720		32	0.0014	
"	0.0040	0.037		380	27		0.0045
3,4-Dihydroxyphenethylamine	0.0000	0.042	510		24	0.0020	
"	0.0040	0.054		230	19		0.0033

Notable in the data of Table VI is the fair agreement between $1/V_{max.}$ values for a given substrate with and without inhibitor, which affords the

simplest test of the competitive nature of the inhibitory process. Some small changes do occur, but they approximate the variations that occur on occasion between different experiments. While the experiments with the amylamine and hydroxytyramine were made with but one enzyme preparation, the experiments with phenethylamine were made with two different preparations, and those with tyramine with another. The difference in $V_{max.}$ between the two experiments with tyramine without inhibitor simply represents the variance in activity of the enzyme preparations.

Even more notable in these experiments are the marked variations in K_i, dependent upon the substrate competed with. While phenisopropylamine is one-third to one-fourth as active an inhibitor for tyramine and hydroxytyramine as for amylamine, it is only one-thirtieth to one-fortieth as active for phenethylamine inhibition.

DISCUSSION

While the data given with regard to the inhibition of amine oxidase oxidation of amylamine as a substrate in fixed concentration do show a large number of compounds to act as inhibitors, the extent to which such data can be taken to indicate expected relationships under other conditions is limited. This is probably particularly so if other substrates are to be considered by analogy to amylamine.

With regard to relationships between structure and inhibition of amine oxidase upon a single substrate, the secondary and tertiary carbinamines are very generally inhibitors. The molecular size of the alkyl and phenylalkyl secondary carbinamines would seem to have some specificity with regard to the inhibitor effect, with α-methylamylamine and α-methylphenylpropylamine appearing to be slightly the most active in their respective series. The generally decreased inhibitor activity of hydroxy derivatives in the side chain or ring of phenethylamines is of most interest in connection with their physiological activities.

The great differences in the inhibitor effect of phenisopropylamine when kinetically valued against particular type substrates show that, while the inhibition exhibited may be an equilibrium situation, it is inadequately considered as an equilibrium only involving $2E + S + I \rightleftharpoons ES + EI$, or it may be that the limiting rate of the reaction is not only the oxidation rate of ES, but also that rates of other reactions are slower than, or comparable to, such oxidation rate. With regard to the equilibrium components, it must be remembered that there are some qualitative rate observations with extracts of liver and other organs of various species that seem to be most easily explained by assuming the presence of several types of amine oxidase enzymes.

The set-up of rate equations for the combination of $E + S \rightarrow ES$, together with the dissociation of $ES \rightarrow E + S$ and the conversion of $ES \rightarrow E + R$ (the oxidized product) for the analysis of all possible rates controlling the kinetics of reaction between enzyme and substrate, has been carried out by Haldane (13). Such analysis could be extended in the present case to include $E + I \rightarrow EI$ and $EI \rightarrow E + I$ as independent rates. By extending rate observations in a systematic way with variance of enzyme, substrate, and inhibitor concentrations independently, such an analysis might be permitted to be made, but we must leave this problem for others.

The increase in oxidation rate observed when 4:1 and 16:1 ratios of phenisopropylamine were used with 0.004 M phenethylamine as substrate appears to be a real effect. It has been noted with certain other secondary carbinamines under some substrate concentrations and particular secondary carbinamine-substrate ratios. The phenomenon has not been investigated, but may be related to the observations of Mann and Quastel (14) who found in brain oxidations with particular substrates that the binding of produced aldehydes by phenisopropylamine caused an over-all increase in oxidation rate. It may be that this effect most notably enters into the lowered inhibitor activity of phenisopropylamine upon phenethylamine as the substrate.

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